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Single chain antibodies (scFv) represent powerful interventional agents for the achievement of targeted therapeutics. The practical utility of these agents have been limited, however, by difficulties related to production of recombinant scFv and the achievement of effective and sustained levels of scFv in situ. To circumvent these limitations, we have developed an approach to express scFv in vivo. An anti-erbB2 scFv was engineered for secretion by eukaryotic cells. The secreted scFv could bind to its target and specifically suppress cell growth of erbB2-positive cells in vitro. Adenoviral vectors expressing the cDNA for the secretory scFv likewise could induce target cells to produce an anti-tumor anti-erbB2 scFv. In vivo gene transfer via the anti-erbB2 scFv encoding adenovirus also showed anti-tumor effects. Thus, by virtue of engineering a secreted version of the anti-tumor anti-erbB-2 scFv, and in vivo expression via adenoviral vector, effective concentrations of scFv were achieved. In vivo gene transfer clearly represents a powerful means to realize effective scFv-based approaches. This method will likely have applicability for a range of disorders amenable to targeted therapeutic approaches.

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Introduction:

Antibody (mAb) based therapy currently offers exciting potentials as a approach to disseminated breast cancer. We have developed a novel approach to achieve anti-tumor mAb therapy via genetic induction of antibody expression.

Proposal Body

Targeted theraputics for carcinoma of the breast are based upon an understanding of the molecular lesions etiologic of neoplastic transformation and progression. In this regard, approaches have been developed to abrogate the effects of overexpression of the erbB-2 oncoprotein. Such interventions have included methods to block expression of the erbB-2 oncogene, via antisense or ribozymes, or methods to block the oncoprotein's contributions to autocrine growth promoting areas, via monoclonal antibody (mAb)-based therapies. This latter approach has been translated into human clinical protocols and has demonstrated efficacy in the context of advanced phase trials. These studies have thus established the concept that mAbs may represent a feasible and effective means to achieve a targeted anti-tumor effect for therapy of carcinoma of the breast. The principal limits to mAb-based therapies relate to achieving adequate levels of anti-tumor agent at sites of disease, and to maintain these effective levels for a sufficient period of time. In this regard, biodistribution and clearance aspects of mAbs frequently mitigate against the maintenance of prolonged and effective levels of mAb at tumor sites. These delivery issues have been addressed by the employment of engineered antibody derivatives including single-chain antibodies (scFvs) and chimeras derived from murine and human mAbs. Despite the improvements offered by these antibody engineering maneuvers, effective delivery remains to be fully achieved. On this basis, methods to circumvent these delivery limitations would allow the realization of the full therapeutic potential of mAbs in the therapy of carcinoma of the breast. In this regard, we have employed gene therapy methods as a means to express anti-tumor single chain antibodies. We have employed these as "intrabodies" to achieve functional knockout of oncoproteins in tumor cells with the achievement of a targeted anti-tumor effect. Indeed, we have translated the intrabody approach into the context of human clinical trial for erbB-2 positive cancers. In addition, we have translated the intrabody approach into the context of a human clinical trial for erbB-2 positive cancers. In addition, we have used these same methods of gene-based eukaryotic scFv expression to achieve secretion of scFv by transduced cells. Such "secretory" anti-erbB-2 scFv maintain the anti-tumor efficacy of the parent antibody in the context of in vitro model systems of erbB-2 positive breast cancer. Of note, genetic transduction offers the means to accomplish prolonged expression of therapeutic proteins as well as the potential to express such proteins with spatial precision. On this basis, gene based expression of secretory scFv theoretically allows a means to address the current limits of mAb based approaches. The realization of this novel method to employ targeted mAbs thus allows a method for breast cancer therapy, which may be readily tested for efficacy in stringent model systems. Anti-erbB-2 mAbs represent of the more promising new agents for carcinoma of the breast in recent decades. Human Trials of mAbs have clearly established the technical restrictions which limit the realization of the full therapeutic value of these agents. Gene-based expression of secretory scFv represents a direct utilization of gene transfer methods to address these technical limits. As such, secretory anti-erbB-2 scFv represent a logical fusion of two technologies. Data relating to our execution is enclosed herein (Appendix A).

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Key Research Accomplishments:
- Demonstration that single chain antibodies (scFv) can be expressed genetically in vivo
- Demonstration that scFv generated in vivo can elicit anti-tumor effects

Reportable Outcomes: *Effective single chain antibody (scFv) concentrations in vivo via adenoviral vector mediated expression of secretory scFv*, Arafat WO, Gomez-Navarro J, Buchsbaum DJ, Xiang J, Wang M, Casado E, Barker SD, Mahasreshti PJ, Haisma HJ, Barnes MN, Siegal GP, Alvarez RD, Hemminki A, Nettelbeck DM, Curiel DT., Gene Ther 2002 Feb;9(4):256-62

CONCLUSION: In vivo transduction provides a novel means to achieve antibody based therapy for cancer of the breast.

APPENDICES: Arafat WO, Gomez-Navarro J, Buchsbaum DJ, Xiang J, Wang M, Casado E, Barker SD, Mahasreshti PJ, Haisma HJ, Barnes MN, Siegal GP, Alvarez RD, Hemminki A, Nettelbeck DM, Curiel DT, *Effective single chain antibody (scFv) concentrations in vivo via adenoviral vector mediated expression of secretory scFv.*, Gene Ther 2002 Feb;9(4):256-62

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APPENDIX A

RESEARCH ARTICLE

Effective single chain antibody (scFv) concentrations in vivo via adenoviral vector mediated expression of secretory scFv

WO Arafat[1,4], J Gómez-Navarro[1,4], DJ Buchsbaum[5], J Xiang[1,4], M Wang[1,4], E Casado[1,4], SD Barker[1,4], PJ Mahasreshti[1,4], HJ Haisma[6], MN Barnes[3], GP Siegal[2,4], RD Alvarez[3,4], A Hemminki[1,4], DM Nettelbeck[1,4] and DT Curiel[1,4]

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Single chain antibodies (scFv) represent powerful interventional agents for the achievement of targeted therapeutics. The practical utility of these agents have been limited, however, by difficulties related to production of recombinant scFv and the achievement of effective and sustained levels of scFv in situ. To circumvent these limitations, we have developed an approach to express scFv in vivo. An anti-erbB2 scFv was engineered for secretion by eukaryotic cells. The secreted scFv could bind to its target and specifically suppress cell growth of erbB2-positive cells in vitro. Adenoviral vectors expressing the cDNA for the secretory scFv likewise could induce target cells to produce an anti-tumor anti-erbB2 scFv. In vivo gene transfer via the anti-erbB2 scFv encoding adenovirus also showed anti-tumor effects. Thus, by virtue of engineering a secreted version of the anti-tumor anti-erbB-2 scFv, and in vivo expression via adenoviral vector, effective concentrations of scFv were achieved. In vivo gene transfer clearly represents a powerful means to realize effective scFv-based approaches. This method will likely have applicability for a range of disorders amenable to targeted therapeutic approaches.
Gene Therapy (2002) 9, 256–262. DOI: 10.1038/sj/gt/3301639

Introduction

The development of antibody-based therapies has allowed the achievement of the first valid targeted therapies for neoplastic disease.[1] Specifically, the direct administration of anti-tumor monoclonal antibodies (mAbs) has shown efficacy in a variety of model systems, and currently has demonstrated definitive benefit in human clinical trials in the context of various lymphoma tumor targets.[2] Based on these promising results, a number of strategies have endeavored to optimize the performance of mAbs for clinical application. In this regard, immune response against murine antibody domains not involved in antigen recognition/binding (HAMA) has led to the development of single chain antibody (scFv) agents.[3] These antibody components, derived via molecular engineering or phage display methods, can embody the full anti-tumor potency of the parent antibody. Furthermore, their size/structure offer some advantages in terms of biodistribution, tumor penetration and immunogenicity.

Despite the potential advantages offered by scFvs, full employment of these agents has been limited by several key factors. In this regard, the principle means for realization of preparative amounts of scFv has been via production in recombinant systems in vitro. Unfortunately, only a minority of candidate anti-tumor scFvs has proven amenable to large scale, high yield production via these methods. Further, biodistribution and metabolism aspects frequently limit the achievement of effective and sustained levels of anti-tumor scFv in the target tissue.[3,4]

On this basis we have explored a novel strategy of employment of scFv for anti-tumor applications. Taking advantage of the capacity of adenovirus vectors to achieve efficient in vivo gene transfer, we have endeavored to express anti-tumor scFvs as secretory molecules in situ. This method appears to offer the means to produce functional scFv via eukaryotic cell synthesis, and to allow for high level and sustained concentrations of scFv, which can achieve anti-tumor efficacy. This in vivo secretory scFv approach may thus allow wider application of the anti-tumor scFv approach via circumventing the key technical and biological limits noted in this context.

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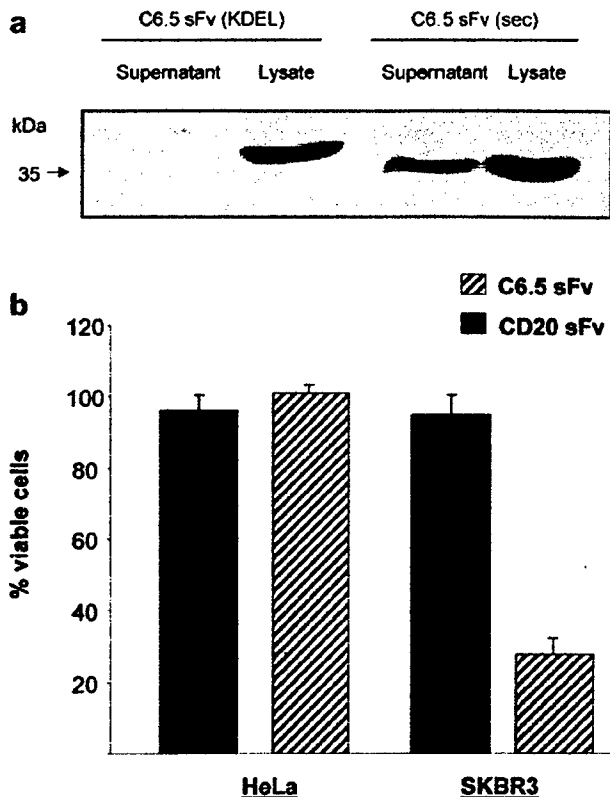
Results

Monoclonal antibodies to erbB-2 have recently been reported to achieve efficacy in the context of human clinical trials.[5] On this basis, we designed a model system to test our concept employing an anti-erbB-2 scFv. For further studies we thus obtained the anti-erbB-2 scFv C6.5, which recognizes an ectodomain of the erbB-2 oncoprotein.[6] The cDNA of the anti-erbB-2 scFv was configured into a plasmid vector pSTCF allowing eukaryotic expression. This vector contains an Igk leader sequence, which routes heterologous proteins via the cellular secretory pathway. As a control, the plasmid pSTCF.KDEL, which contains an endoplasmic reticulum (ER) retention signal, was employed to allow derivation of non-secreted versions of the tested scFvs. Trial studies utilized the anti-erbB-2 scFv cDNA of C6.5, plus a control scFv, directed against a non-erbB-2, B cell antigen CD20. In addition, the expression plasmids contained a carboxy-terminal myc tag to allow analysis of expressed transgene products. HeLa cells transfected with the ER-retained form of the control anti-erbB-2 scFv expressed protein only in cellular lysate. In contrast, deletion of the KDEL retention signal allowed secretion of the scFv, whereby it could be readily detectable in the supernatant (Figure 1a). These studies thus establish that eukaryotic expression of the anti-erbB-2 scFv could be achieved such that the molecule was routed via the cellular export apparatus with the achievement of a secreted form.

Anti-erbB-2 mAbs can achieve direct cytotoxic effects selectively against erbB-2 overexpressing tumor targets.[7] We thus wished to validate that our secreted anti-erbB-2 scFv retained its specific anti-tumor activity. For these studies, supernatants from HeLa cells transfected with the plasmids expressing the secretory forms of the anti-erbB-2 scFv, or the control anti-CD20 scFv, were incubated with erbB-2-negative HeLa or erbB-2-positive SKBR3 cells. Direct determination of cell viability demonstrated that the supernatants deriving from the secretory anti-erbB-2 scFv exhibited a selective cytotoxicity for the SKBR3 cells not observed in the context of the anti-CD20 scFv control (Figure 1b). Whereas erbB-2-binding-ligand interactions exert biologic effects via diverse effector mechanisms,[8] for C6.5, direct binding to the erbB-2 target appears necessary, and sufficient. Of note, the results confirm that an anti-erbB-2 scFv, derived via eukaryotic cell secretion, can retain its specific cytotoxic activity.
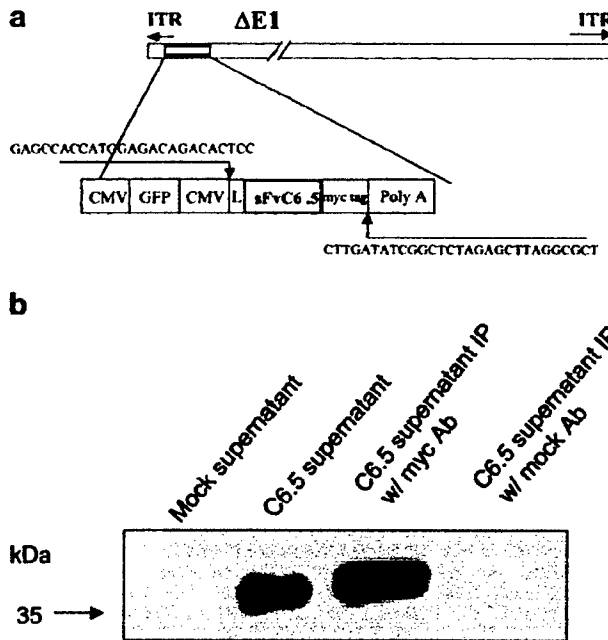
On this basis we sought to develop a vector approach which would allow *in vivo* expression of the scFv for therapeutic application. In this regard, recombinant adenoviral vectors (Ad) achieve unparalleled levels of *in vivo* gene transfer in the context of a variety of end organ targets.[9] We thus configured the cDNA encoding the secretory forms of the anti-erbB-2, and anti-CD20 scFvs, into adenoviral vectors to allow high level *in vivo* expression. These vectors were replication-defective adenoviruses whereby the transgene was configured into the deleted E1 site (Figure 2a). Further, these viruses were capable of enhanced infectivity by virtue of an RGD4C peptide configured within the HI loop of fiber knob.[10,11] Infection of 293 cells and analysis of cellular supernatants confirmed that the Ad vectors were capable of achieving detectable levels of the secretory anti-erbB-2 scFv (Figure 2b).

We next sought to confirm the biologic activities of the

vector-induced secretory scFvs. The initial analysis sought to confirm specific direct binding of the secreted scFv to its cognate on target cells. For these studies adenoviral vectors encoding either the anti-erbB-2 or anti-CD20 secretory scFvs were employed to infect the erbB-2 negative cell line HeLa and derived cell supernatants were then administered to the erbB-2-positive ovarian cancer cell line SKOV3.ip1 or erbB-2-negative cell line HeLa. Treated cells were then harvested for Western blot analysis of total cellular lysate via antibody to the myc tag. In this study, it could be noted that the secretory erbB-2 exhibited specific binding exclusively to the erbB-2 positive target cells (Figure 3a). As a further confirmation, immunofluorescent analysis was performed employing the anti-myc-tag antibody. This analysis demonstrated distinct patterns of staining for the erbB-2-posi-

Figure 1 Detection and selective cytotoxic effect of the secretory C6.5 single-chain antibody (a) HeLa cells were transfected with myc-tagged secretory (pSTCF.C6.5) or a non-secretory version (pSTCF.KDEL.C6.5) of the anti-erbB-2 single-chain antibody C6.5 (a). After 48 h, supernatant and cell lysates were collected separately. The supernatant was immunoprecipitated with an anti-myc-tag antibody. The whole lysate and the whole immunoprecipitated supernatant were analyzed by SDS-PAGE in a 12% gel, followed by Western blotting with a monoclonal anti-myc-tag primary antibody. (b) HeLa cells were transfected with plasmids encoding the secretory versions of either the anti-erbB-2 single-chain antibody C6.5, pSTCF.C6.5, or the control anti-CD20 single-chain antibody, pSTCF.CD20. After 48 h, supernatant was collected. Then, erbB-2-positive cells (SKBR-3) and erbB-2-negative cells (HeLa) were treated with those supernatants containing anti-erbB-2 or anti-CD20 single-chain antibodies. An MTS assay was performed 4 days later to measure cell survival. Experiments were performed in triplicates and results are indicated as mean ± s.e.

a

b

Figure 2 Detection of single-chain antibody in the supernatant of cells infected with an adenovirus encoding the anti-erbB-2 secretory scFv. A recombinant adenovirus vector encoding the secretory version of C6.5 was constructed (L, leader; myc, myc tag). (a) Map of recombinant adenovirus vector. A double expression cassette inserted into the deleted E1 region which contains GFP and C6.5 with myc-tag signal, each driven by the CMV promoter and followed by a polyadenylation signal. (b) 293 cells (erbB-2 negative) were infected with the recombinant adenovirus encoding the myc-tagged anti-erbB-2 secretory scFv, RGD.Ad.C6.5, or mock infected. After 48 h, supernatant was collected and immunoblotted, or immunoprecipitated, with anti-myc-tag antibody. Whole immunoprecipitated supernatant was analyzed by SDS-PAGE in a 12% gel, followed by Western blotting with monoclonal primary anti-myc-tag antibody.

a

b
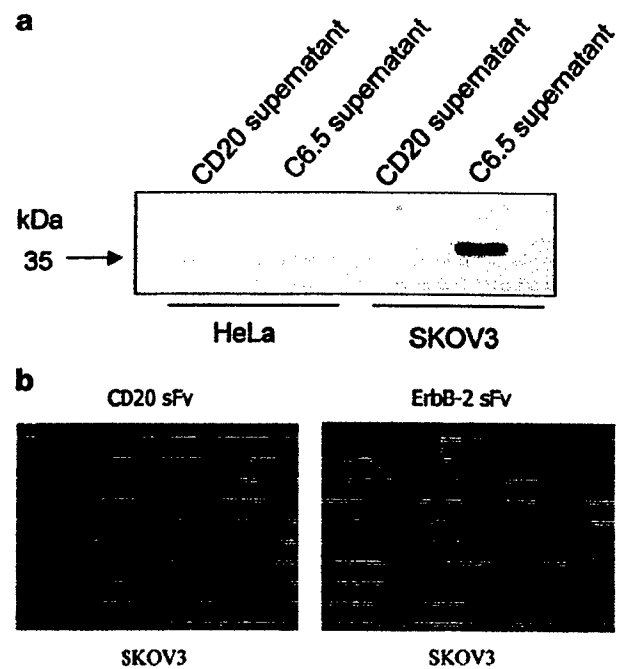
Figure 3 Specific binding of the secretory anti-erbB-2 single chain antibody to erbB-2-positive cells. (a) HeLa and SKOV3.ip1 cells were incubated for 1 h at 4°C with supernatant containing either the C6.5 single-chain antibody or the control, anti-CD20. Cell lysate was then harvested and analyzed by SDS-PAGE in a 12% gel, followed by Western blotting with a monoclonal primary anti-myc-tag antibody. (b) SKOV3.ip1 cells were also incubated with supernatant containing anti-CD20 or C6.5 single-chain antibodies for 4 h at 37°C. Cells were then washed with PBS and fixed with methanol. Then, cells were incubated with anti-my-tag antibody. Indirect immunofluorescent microscopy analysis for the presence of the anti-erbB-2 single-chain antibody is shown.

tive cellular target in the context of the secretory anti-CD20 and anti-erbB-2 scFvs (Figure 3b). Specifically, the anti-erb-2 scFv achieved a homogenous pattern consistent with specific surface binding to target cells. These studies thus confirmed that the adenoviral vector-mediated expression of the anti-erbB-2 scFv allowed the derivation of a secreted form of the scFv, which retained the capacity for selective target cell recognition.

We next studied the cytotoxic properties of the secretory anti-erbB2-scFv derived via adenoviral vector expression. For this analysis, supernatants of cells infected with the secretory anti-CD20 or anti-erbB-2 scFv encoding adenoviral vectors, or of uninfected cells, were incubated with the erbB-2-positive target cell line SKOV3.ip1. At various points after administration, cell viability was determined. In these studies it could be seen that the anti-erbB-2 scFv-derived via Ad expression achieved a direct, specific anti-tumor effect (Figure 4). These studies thus confirm that the secretory anti-erbB-2 derived via Ad expression exhibited the key capacities relevant to its employment for anti-tumor therapeutics.

Adenoviral vectors can achieve effective *in vivo* gene transfer after systemic vector administration.[9] We thus hypothesized that delivery of the vector encoding the
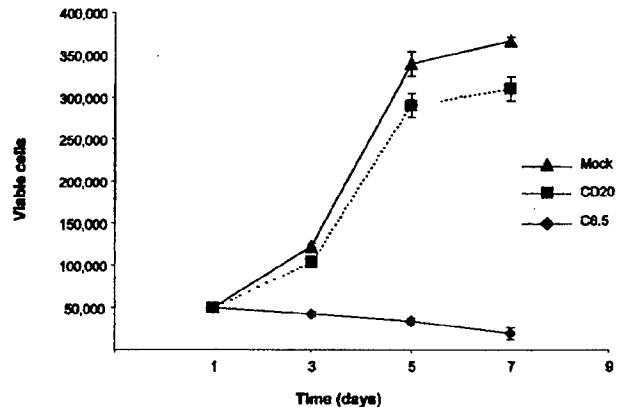
Figure 4 Specific cytotoxic effect of the secretory anti-erbB-2 single-chain antibody on erbB-2 overexpressing cells. HeLa cells (erbB-2 negative) were infected with recombinant adenoviruses encoding the secretory versions of the anti-erbB-2 (RGD.Ad.C6.5), or anti-CD20, (RGD.Ad.CD20) single-chain antibodies, or with normal media. After 48 h, supernatant was collected and SKOV3.ip1 cells (erbB-2-positive) were treated with the supernatant. Cell proliferation over 7 days was measured by MTS assay. Experiments were performed in triplicates and results are indicated as mean ± s.e.

secretory anti-erbB-2 scFv would allow the achievement of *in situ* infection with the consequent induction of effective scFv expression. Further, owing to the capacity of the delivered scFv to achieve secretion via eukaryotic cells, serum levels of the anti-erbB-2 scFv should be obtained. We thus administered the adenoviral vector encoding the secretory anti-erbB-2 scFv via the tail vein of mice and analyzed the serum from treated animals for the presence of the anti-erbB-2 scFv, employing an ELISA to human immunoglobulin. In this analysis, clearly detectable levels of the induced scFv were observed within the serum of test animals (Figure 5a). Of note, the magnitude of the secretory scFv was relatively sustained, with clearly detectable levels visible through the period of 10 days.

This expression pattern is consistent with previous reports of heterologous gene expression induced by *in vivo* Ad administration, whereby heterologous transgene products may be detected for periods of up to 2 3 weeks.[12]

The achievement of high and sustained levels of the anti-erbB-2 scFv within the serum of test animals provided the basis for *in vivo* tumor challenging studies. For these experiments, we employed a xenograft model, whereby a human ovarian cancer cell line engraftment was established in the context of athymic nude mice. Test animals were treated with Ad vectors encoding the secretory forms of the anti-CD20, or anti-erbB-2, scFvs, and challenged with tumor 48 h later by subcutaneous injection of the erbB-2-positive tumor cell line SKOV3.ip1. Whereas exponential growth in tumor volumes was noted in the two control groups, a clear inhibition of tumor growth was observable for the animals treated with the Ad vector encoding the secretory anti-erbB-2 scFv (Figure 5b). These studies thus clearly establish that anti-tumor scFv is expressed at effective concentrations *in situ* via adenoviral vector mediated *in vivo* gene delivery. Clearly, design issues of timing and magnitude may be capable of achieving an even more profound inhibition of tumor growth.

Discussion

Genetic engineering of antibodies has been utilized in a variety of ways to address the limitations noted with direct employment of mAbs.[1] In this regard, genetic modifications of mAbs have allowed derivation of scFvs as a means to reduce the human immune response directed against the mAb, as well as to improve biodistribution.[3] Here we employ genetic engineering of antibodies to address a distinct aspect of mAb use. Specifically, we have exploited the ability to achieve *in vivo* gene delivery to achieve scFv expression *in situ*. The genetic expression of the scFv has allowed prolonged presence of the scFv, which thereby allowed the achievement of an anti-tumor effect. On this basis, *in vivo* secretion of scFvs represents a novel application which may allow distinct advantages.

Specifically, a significant fraction of candidate scFvs cannot be provided at preparative magnitude via available prokaryotic and eukaryotic systems. The ability to express the scFv after *in vivo* genetic transduction may circumvent this limit and thus allow production of a range of useful scFv at effective concentrations. In this regard, Whittington *et al*[13] employed a murine system to
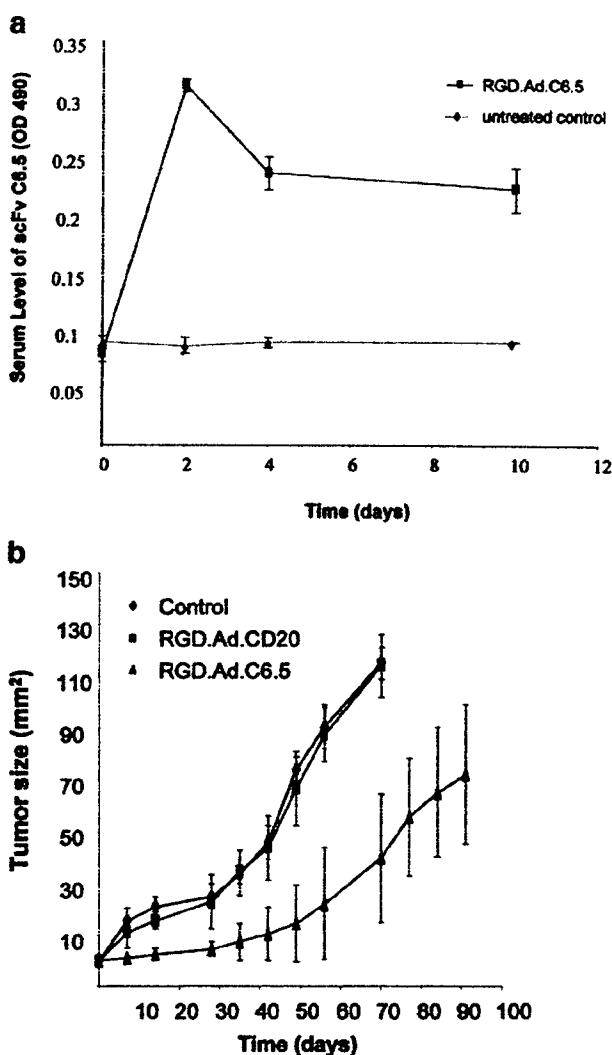
Figure 5 Detection of secretory single-chain antibody in the serum of mice and inhibition of tumorigenesis in a mouse model of erbB-2-positive cancer cells after systemic administration of RGD.Ad.C6.5. (a) Athymic nude mice were injected via the tail vein with 5×10^9 pfu of the recombinant adenovirus encoding the secretory version of the anti-erbB-2 single-chain antibody, RGD.Ad.C6.5, or left untreated. Blood samples were collected from treated and untreated animals up to 10 days after injection. Serum was analyzed by ELISA for the presence of the human C6.5 single-chain antibody using an anti-human IgG antibody. Results are indicated as mean ±s.e. (b) Athymic nude mice were injected via the tail vein with 5×10^9 pfu of the recombinant adenovirus encoding the secretory version of the anti-erbB-2 single-chain antibody, RGD.Ad.C6.5, or with control virus. After 48 h, 2.5×10^6 SKOV3.ip1 cells were injected subcutaneously (2 injections per mouse). Mice were monitored for tumor formation and size for a period of 8 weeks. Results are indicated as mean ±s.e. Tumor growth inhibition by RGD.Ad.C6.5 was statistically significant relative to the controls (P = 0.0001).

produce an scFv-fusion not readily produceable in conventional systems. Additionally, *in vivo* transduction may allow production of a large magnitude of scFv for a more prolonged interval than obtainable via direct systemic administration of recombinant scFv (the latter with a half-life of only a few hours in blood). Indeed, the

dynamics of biosynthesis and secretion from a variety of other target organs such as muscle could potentially offer even greater prolongations of expression noted here. Furthermore, adenovirus/AAV and adenovirus/retrovirus chimeras may combine efficient adenovirus-mediated gene transfer with stable gene expression after chromosomal integration of the scFv transgene by AAV or retroviral sequences.[14] Finally, the ability to target gene delivery to selected sites may offer the means to express therapeutic scFv directly at points of target intervention. The employment of genetic engineering methods allowed the realization of scFv from mAb with the accrual of key application benefits. In addition, similar strategies may provide a means to effectively express antibody-based therapeutic agents such as immunotoxins.[15] The further use of gene therapy methods, as shown here, may offer further benefits in the context of targeted antibody therapeutics by allowing further advances in terms of the achievement of prolonged and effective concentrations of therapeutic scFv.

Materials and methods

Cell culture
The human ovarian carcinoma cell line SKOV3.ip1 was kindly provided by Dr Janet Price (University of Texas, MD Anderson Cancer Center, Houston, TX, USA). The human cell lines SKBR-3 (breast carcinoma), 293 (embryonic kidney), and HeLa (cervical carcinoma) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in DMEM/F12 media (SKOV3.ip1, 293, and HeLa) or McCoy s media (SKBR-3) supplemented with 10% fetal calf serum, 200 µg/ml L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Plasmid construction and preparation
The anti-erbB-2 scFv C6.5 and anti-CD20 scFv cDNAs[16] were cloned into the plasmid pSTCF.KDEL giving rise to the plasmids pSTCF.KDEL.C6.5 and pSTCF.KDEL.CD20. For cloning strategy see Arafat *et al*.[17] The expression plasmid pSTCF.KDEL was derived from pSecTagC (Invitrogen, San Diego, CA, USA) as previously described.[17] This plasmid contains an Igκ leader sequence that targets encoded proteins to the secretory pathway, followed by a polylinker, an in-frame myc-tag sequence and a KDEL endoplasmic reticulum (ER) retention signal sequence leading to retention of the encoded scFv in the ER. pSTCF plasmids encoding the secretory anti-erbB2 scFv C6.5 (pSTCF.C6.5) and the secretory scFv CD20 (pSTCF.CD20) were constructed by deleting the KDEL fragment of pSTCF.KDEL.C6.5 and pSTCF.KDEL.CD20.

Plasmid transfection
Transfections were performed using the adenovirus/polylysine system (Ad/pL), as previously described.[18] Briefly, 6 µg of plasmid DNA was conjugated with an optimized amount of AdpL vector and 4 µg of free poly-L-lysine, constituting one complex. Complexes were diluted in culture medium containing 2% FCS and added to cells in six-well plates. After 1 h, an equal volume of complete medium was added to the wells.

Virus construction and validation
Adenovirus vectors encoding the secretory scFvs were constructed by the AdEasy method.[19] To this end, scFv cDNAs were cloned into the pTrackCMV plasmid.[19] The resulting plasmids contained in tandem the CMVie promoter-driven gene encoding the secretory scFv, as well as the CMVie promoter-driven GFP reporter gene. Homologous recombination of these plasmids with pVK503[20] gave rise to the RGD.Ad.C6.5 and RGD.Ad.CD20 adenoviral genomes. The corresponding viruses were rescued by transfection of 293 packaging cells. These viruses contained a RGD4C-peptide incorporated into the HI-loop of the fiber knob capsid protein resulting in increased infection efficiency for several cell types.[20] Virus preparations were characterized by restriction analysis, PCR and sequencing. Viral particle (vp) and infectious particle concentrations were determined by OD260 and plaque assay (pfu), respectively, using standard techniques. Titers for RGD.Ad.C6.5 were 5×10^{11} vp/ml and 2×10^{10} pfu/ml; those for RGD.Ad.CD20 were were 7×10^{11} vp/ml and 4×10^{10} pfu/ml.

Adenoviral infection
Cells were infected with recombinant adenoviruses at a MOI of 100 pfu/cell in DMEM, 2% heat-inactivated FCS for 2 h. Then, cells were washed and the medium was replaced by normal medium.

Immunoprecipitation
Supernatant of cells transfected with plasmids pSTCF.KDEL.C6.5, pSTCF.KDEL.CD20, pSTCF.C6.5, or pSTCF.CD20, or of cells infected by the viruses RGD.Ad.C6.5 or RGD.Ad.CD20 or of untreated cells, was collected and immunoprecipitated with a polyclonal rabbit anti-myc-tag antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To this end, the antibody was mixed with 500 µl of immunoprecipitation (IP) wash buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM sodium vanadate, 1% Triton X-100, 10% glycerol), 500 µl of gold buffer (2 mM Tris, 150 mM NaCl, gelatin, 0.1% BSA, 0.5% Tween-20, 0.02% sodium azide), and 30 µl of protein A/G plus agarose beads (Santa Cruz Biotechnology), and rotated overnight at 4°C. The beads were then washed by brief centrifugation, followed by resuspension in 1 ml of IP wash buffer. This step was repeated five times. The beads were then resuspended with 500 µl of the cell lysate and rotated at 4°C for 2 h. After washing as above, beads were resuspended in 100 µl of 1 × SDS-PAGE sample buffer and boiled for 10 min. The samples were then evaluated by SDS-PAGE and immunoblot as described below.

Immunoblot analysis
Cells were lysed with a NP-40 buffer containing 0.3% NP-40, 142 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 20 mM HEPES pH 7.4, and a cocktail of protease inhibitors. Total protein (10 50 µg) was separated on a 12% SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane (BioRad, Hercules, CA, USA). The membranes were blocked with 2% non-fat dry milk. Membranes were probed with the anti-myc-tag antibody 9E10 (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:2500, for scFv detection. After washing with TBS containing 0.05% Tween-20, a secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA,

USA) 1:3000 diluted in TBST was added for a 1-h incubation. Finally, the blot was washed and developed using the enhanced chemiluminescence kit (Renaissance; NEN, Boston, MA, USA) according to the manufacturer s protocol.

Cellular cytotoxicity

The effect of expression of the anti-erbB-2 scFv on cell growth and viability was determined employing the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation/Cytotoxicity assay (Promega, Madison, WI, USA). This MTS-based colorimetric assay measures the ability of viable cells to convert a tetrazolium salt to the formazan compound. HeLa cells were transfected with plasmids encoding secretory scFv C6.5 or secretory scFv CD20, or infected with adenovirus encoding the same proteins as described above. Supernatants were collected after 48 h. For the cytotoxicity assay, HeLa, SKBR-3 or SKOV-3.ip1 cells were seeded into 96-well cell culture plates at a density of 5000 cells/well. Twenty-four hours later, supernatant containing scFv C6.5 or scFv CD20 was transferred. The plate was subsequently incubated for up to 96 h. The Cell Titer 96 kit was then used according to the manufacturer s instructions and absorbance at 490 nm determined. Percent viability was calculated by the following formula: [(absorbance infected cells − blank)/(absorbance uninfected cells − blank)] × 100. To quantify cell numbers from the experimental results, a standard curve was generated by plating cells at defined concentrations in triplicate wells on the day of the assay.

Binding assays

Cells (SKOV3ip1 and HeLa) were treated with medium containing scFv C6.5, irrelevant scFv (CD20) or left alone for 1 to 6 h at 4°C. Subsequently, cells were harvested, washed three times with PBS and subjected to immunoblot as described above.

Immunofluorescence assay

Cells plated in a two-well chamber slides (Nalge; Nunc, Naperville, IL, USA) at a density of 100 000 cells/well were treated with medium containing scFv C6.5 or scFv CD20 for 1 to 6 h at 4°C. Medium was removed and cells were washed twice with PBS, fixed with methanol for 5 min at room temperature and washed five times with PBS. After blocking with PBS/FBS 10% for 15 min at room temperature, cells were incubated for 20 min with anti-myc-tag antibody (Invitrogen) diluted 1:500 in PBS. After washing twice with PBS, secondary FITC-labeled anti-mouse antibody diluted 1:1500 in PBS/FBS 10% was incubated on the cells for 20 min in the dark. Next, cells were washed twice with PBS, stained with Hoechst 33258 diluted 1:1760 in PBS for 10 min, washed with PBS, mounted on coverslips and analyzed with an Olympus IV fluorescence microscope (Olympus, Tokyo, Japan).

ELISA for detection of scFv in blood

Nude mice, eight mice per group, were injected into the tail vein with 5×10^9 pfu of RGD.Ad.C6.5 or RGD.Ad.CD20 or left untreated. Blood was collected from the animals at 0, 24, 48, 96 h, 10 and 15 days after virus injection into a microcontainer serum separator tube (Becton Dickinson, Franklin Lakes, NJ, USA). Serum was then separated by centrifugation at 1000 r.p.m. for 10 min. Serum was added to an ELISA plate at concen-

trations of 200 ng protein per well and left at 4°C overnight. Wells were blocked using 1% BSA/TBA buffer for 1 h and washed three times with TBS. Then, goat anti-human antibody conjugated to alkaline phosphatase (Pierce, Rockford, IL, USA) was added and incubated overnight at 4°C. Wells were washed five times with TBS. Subsequently, substrate was added and colorimetric measurements were performed at 490 nm wavelength.

Inhibition of tumorigenesis in vivo

Nude mice, eight mice per group, were injected into the tail vein with 5×10^9 pfu of RGD.Ad.C6.5 or RGD.Ad.CD20 or left untreated. Forty-eight hours later, animals were challenged for tumor formation by subcutaneously injecting 2.5×10^6 SKOV3.ip1 cells. Mice were monitored for tumor nodule formation up to 14 weeks. Tumor diameters were measured every 3 days using a Vernier caliper, and the surface area (product of length × width) was calculated. Statistical analysis was performed using an unequally spaced repeated measures mixed model utilizing a spatial partial law covariance structure.

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